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Synthesis and Evaluation of Neutral Phosphate Triester Backbone-Modified siRNAs

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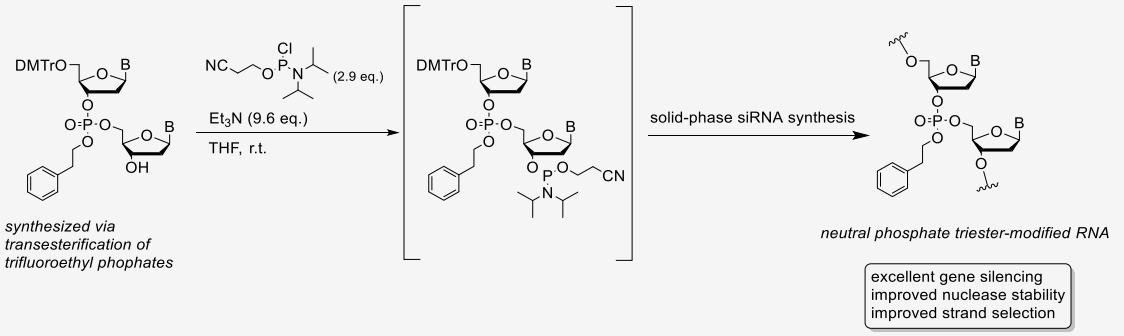

ABSTRACT: Two unsymmetrical dinucleotide phosphate triesters were synthesized via transesterification from tris(2,2,2-trifluoroethyl) phosphate. The protected triesters were phosphytilated to generate phosphoramidites for solid-phase oligonucleotide synthesis. Neutral phenylethyl phosphate-modified short-interfering RNAs (siRNAs) were synthesized and evaluated for their gene-silencing ability, siRNA strand selection, and resistance to nucleases. These backbone-modified phosphate triester siRNAs offer many improvements compared to natural unmodified siRNAs.

KEYWORDS: *Short interfering RNA, backbone modification, phosphate triester, off-target effect*

Short-interfering RNAs (siRNAs) are gaining traction as the next generation therapeutics due to recent successes in the field.[1,2] siRNAs are gene-silencing molecules that target the mRNA with high specificity.[3,4] In order for this to occur, one of the strands from the duplex is loaded into the RNA-induced-silencing-complex (RISC) to form an active RISC-guide-RNA complex, which directly targets the mRNA of interest.[5] With the recent US FDA approval of Patisiran and Givosiran, which target rare diseases, and more RNA-based therapeutics in clinical trials, confidence and optimism in oligonucleotide-based therapeutics is strong.[6] However, these approved siRNAs contain several older generation modifications, namely the 2′-fluoro and 2′-*O*-methyl modifications.[7,8] Despite this recent success, the future of siRNA development toward more common diseases such as cancer depends on expanding the chemical subset for modifications. This is necessary in order to overcome some persistent challenges in siRNA design.

The natural negatively charged phosphodiester backbone of RNA is beset with poor cell-membrane permeability and is a substrate for ubiquitous ribonucleases, which rapidly degrade the RNA to monomers and/or short oligomers.[9] Many different types of backbone modifications have been reported for siRNAs.[10] The most common type is the phosphorothioate backbone, which has seen utility with not only siRNAs but also other therapeutic oligonucleotides such as antisense oligonucleotides.[11] This modification, along with other backbone derivatives, such as boranophosphates, still retains the negative charge.[12] Neutral backbone alternatives such as amides, triazoles, and other noncharged functional groups have been synthesized and studied within siRNAs.[13−15] However, one group that has not been particularly well researched are neutral triester phosphate backbones as a phosphodiester mimic. Some previous groups have reported the synthesis and utility of alkyl phosphate backbone derivatives of oligonucleotides.[16−20] However, these types of studies are scarce, and these types of triester modifications have not seen use within siRNA applications.

In this study, we synthesized a small library of siRNAs bearing a neutral modified phenylethyl phosphotriester back-

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bone on the passenger strand of siRNA duplexes (Figure 1). These siRNAs exhibit potent gene silencing and some offer

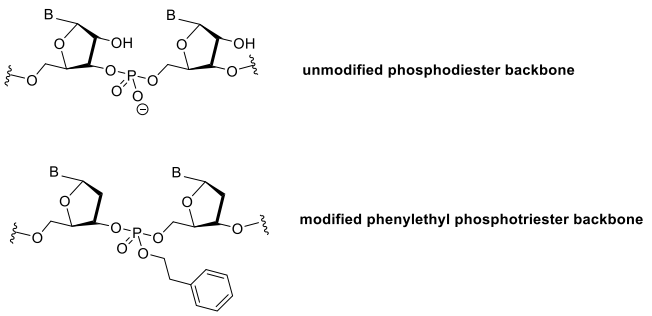
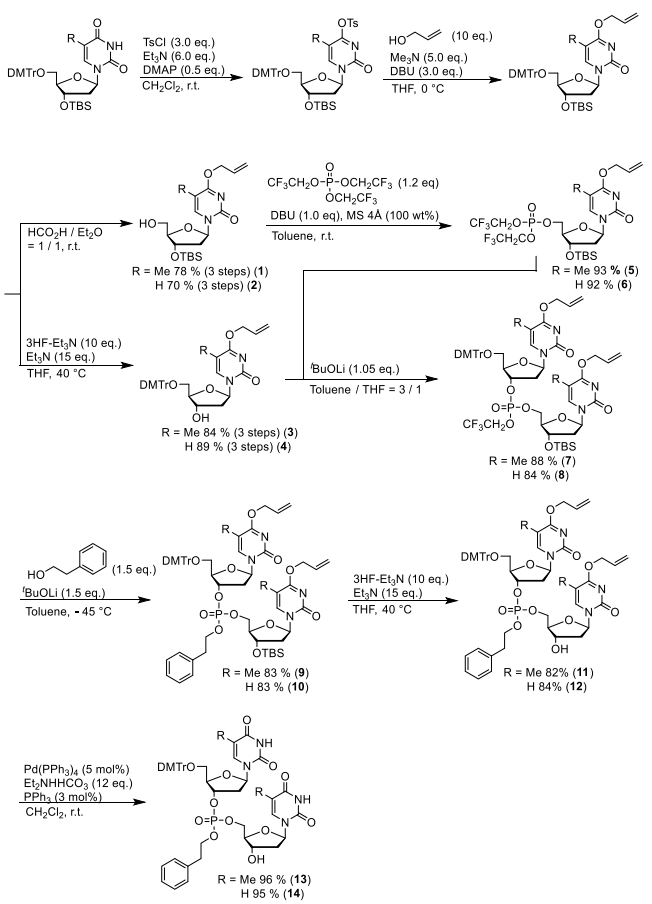


Figure 1. Chemical structural difference between the natural phosphate backbone of RNA (top) and the modified phenylethyl phosphotriester backbone used in this study (bottom).

enhanced target specificity compared to unmodified siRNAs. Furthermore, these modifications offer resistance to nucleases as measured by nuclease stability assays.

To synthesize siRNAs with a neutral phenylethyl phosphotriester backbone, we utilized our recently published methodology (Scheme 1).[21] Our efforts were focused on synthesizing a 4,4′-dimethoxytrityl (DMT)-protected pyrimidine dinucleotide, with a free alcohol. We chose to use a deoxynucleoside in our design rather than a ribonucleoside in order to reduce the number of synthetic steps and because deoxynucleotides have

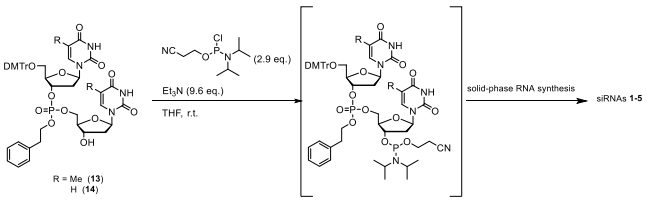
Scheme 1. Synthesis of DMT-Protected Phenylethyl Phosphotriester Dinucleotides



been observed to be a useful modification within siRNA design.[22,23] Due to the acidic proton (NH) of the nitrogenous bases interfering with the transesterification of the phosphate trifluoroethyl ester, we protected the *O*-4 of the pyrimidine heterocycles to solve this problem. First, allyl protected deoxythymidine and deoxyuridine **1**−**4** were prepared according to Noyori's protocol.[24] Next, both *tert*-butyldimethylsilyl (TBS)-protected thymidine derivative **1** and 2′-deoxyuridine derivative **2** were treated with tris(2,2,2-trifluoroethyl) phosphate in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), respectively, and the monosubstituted phosphate triesters **5** and **6** were obtained via selective transesterification in high yield. Then, the monosubstituted phosphate triester **5** was treated with lithium alkoxides generated *in situ* with the DMT-protected thymidine derivative **3** and ^{t}BuOLi. The reaction proceeded selectively, and the desired dinucleotide derivative **7** was obtained in high yield. The dinucleotide derivative **8** was obtained selectively from uridine derivatives **4** and **6** by a manner similar to that of **7**. The transesterification between dinucleotide derivatives **7** and **8** and lithium phenylethoxide afforded allyl protected phosphotriester dinucleotides **9** and **10** respectively in high yield. Deprotections of the TBS group with 3HF·Et_3N and the allyl groups with a palladium catalyst ($Pd(Ph_3)_4$) gave the expected phosphotriester dinucleotide monoalcohols **13** and **14**.

Once the monoalcohols **13** and **14** were formed, phosphoramidites from **13** and **14** were attempted by reaction with 2-cyanoethyl diisopropylchlorophosphoramidite in the presence of triethylamine in THF (Scheme 2).[25] However,

Scheme 2. Synthesis of DMT-Protected Phenylethyl Phosphotriester Dinucleotide Phosphoramidites



both these phosphoramidites were unstable and could not be purified using standard methods. As such, a short column was used to separate unreacted phosphitylating reagent, and the solutions containing the intermediate were immediately used for solid-phase RNA synthesis.

During the synthesis, we used commercially available phenoxyacetyl (PAC) and TBS-protected phosphoramidites so we could use ultramild conditions to deprotect the RNA from the resin (30% aqueous NH_4OH for 2 h at room temperature followed by HF for 2.5 h). This was important so we would not get loss of the phenylethyl group via elimination via standard conditions (for example, overnight deprotection of benzoyl protecting groups with an EMAM solution [1:1 methylamine 33% wt in ethanol and methylamine 40 wt %]). Table 1 highlights the sequences with the modifications that we generated. We purified the sequences by HPLC and confirmed their masses by qTOF-MS (quadrupole time-of-flight mass spectroscopy). To verify that the sequences adopted typical A-form helices, we performed circular dichroism (CD) on the duplexes. CD spectrometry confirmed the formation of the A-form helices (see Supporting

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Table 1. Sequence of siRNAs[a] and Melting Temperatures (T_m)

siRNA	siRNA sequence	T_m (° C)
wt	5′-CUUACGCUGAGUACUUCGAdTdT-3′	74.1
	3′-dTdTGAAUGCGACUCAUGAAGCU-5	
1	5′-**CdUxdU**ACGCUGAGUAC**dUxdU**CGAdTdT-3′	71.8
	3′-dTdTGAAUGCGACUCAUGAAGCU-5	
2	5′ - CUUACGCUGAGUACUUCGA**dUxdU** - 3′	74.4
	3′-dTdTGAAUGCGACUCAUGAAGCU-5	
3	5′-CUUACGCUGAGUACUUCGA**dUxdU**dT-3′	77.1
	3′-dTdTGAAUGCGACUCAUGAAGCU-5	
4	5′-CUUACGCUGAGUACUUCGA**dTxdT**-3′	73.4
	3′-dTdTGAAUGCGACUCAUGAAGCU-5	
5	5′-CUUACGCUGAGUACUUCGA**dTxdT**dT-3′	75.1
	3′-dTdTGAAUGCGACUCAUGAAGCU-5	

[a]**dTxdT** corresponds to the deoxythymidine-based modification; **dUxdU** corresponds to the deoxyuridine-based modification. The top strand is the passenger strand; the bottom strand is the guide strand. The guide strand is 5′-phosphorylated.

Information Figure S-1). In addition, we examined their thermal stability by measuring their melting temperature (T_m). In general, no large changes in T_m were observed compared to **wt** siRNA. This suggests that the impact of the phosphotriester backbone modification on the duplex's thermal stability is minimal.

We designed sequences that target firefly luciferase. Briefly, expression plasmids individually coding for firefly and *Renilla* luciferase (internal control), and the desired siRNA was incubated with lipofectamine, and the mixture was transfected to mammalian cells. Cells were then lysed 24 h post transfection and the efficacy of the siRNAs was evaluated using the Dual-Luciferase Reporter Assay (Promega) system.

siRNA **1** contains the modifications at two internal locations within the passenger strand. As shown in Figure 2, excellent dose-dependent gene silencing is observed with siRNA 1 exhibiting an IC_{50} of 66.9 pM. Compared to wild-type siRNA (wt), this exhibits a small loss of potency (IC_{50} = 5 pM). However, this dose-dependent knockdown is comparable to other reports with internal neutral backbone modifications.[26,27]

SiRNAs **2**−**5** all exhibit excellent dose-dependent gene-silencing within the wild-type siRNA (**wt**) range (IC_{50}s ranging from 3.5−22.9 pM, see Figure S2 in the Supporting Information). Therefore, these phenylethyl phosphotriester backbone modifications are suitable substrates for the RNA interference pathway.

We next examined the impact of the phenylethyl phosphotriester modification on RISC strand selection through the characterization of select siRNAs. We recently published a paper outlining a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) method to quantify the relative amounts of guide- and passenger-mediated gene-silencing of our siRNA.[30] In unmodified siRNA, the guide strand is preferentially incorporated into the RISC (by siRNA design) due to the preference for (1) nucleotide preference for A or U at the 5′ end and (2) the strand with lower 5′ thermodynamic stability.[5,28,29] While these considerations lower passenger strand uptake, the risk of passenger strand associated off-target effects encourages the development of siRNA with further improved selectivity. Therefore, this assay was used to compare the strand selection of **wt** siRNA and modified siRNAs **1**−**3** developed in this study. As expected, the guide strand was significantly more active in **wt** siRNA than the passenger strand (Figure 3). At 8 pM, the guide strand reached a maximum silencing plateau of ∼75%, whereas the passenger strand required up to 800 pM to exert the same effect. The addition of the phenylethyl triphosphate modifications to **wt** siRNA, as seen in siRNAs **1**−**3**, significantly altered the activity of the two strands. In agreement with the dual-luciferase reporter assays, the guide strand of siRNAs **1**−**3** showed reduced activity with **1** being the least active. Remarkably, when compared to **wt** siRNA, guide strand selection was enhanced for siRNA **1** but diminished for siRNAs **2** and **3**. We predict that the improved selection of siRNA **1** is due to the inability of the nonionic phenylethyl triphosphate to mimic the natural charged backbone interactions that occur between the phosphate and amino acid residues of the RISC.[31] In agreement with previous research using this assay, heightened selection of the passenger strand is associated with chemical modifications at the 3′ terminal of the passenger strand.[30] The ability for 3′

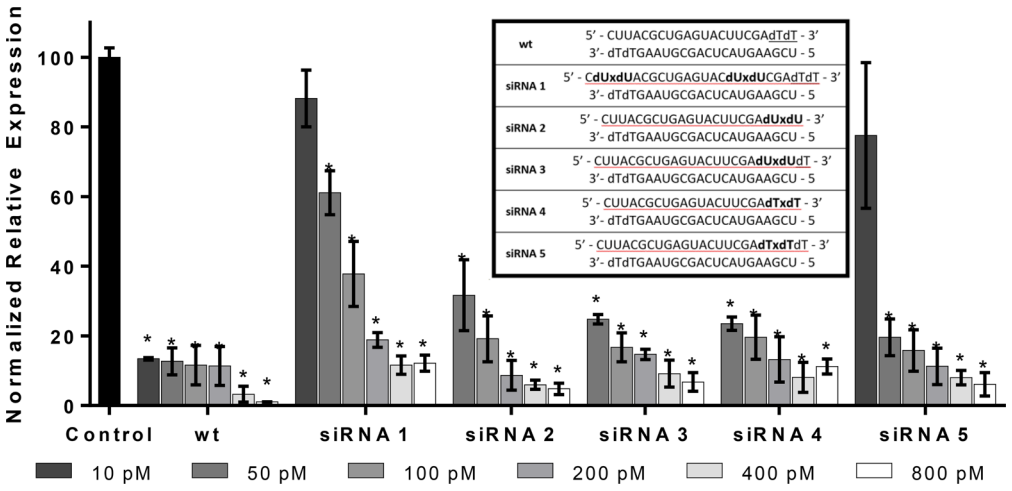

Figure 2. Reduction in firefly luciferase protein expression related to the potency of phenylethyl phosphate-modified siRNAs using the dual-luciferase reporter assay. The siRNAs were tested between 10 and 800 pM, with firefly luciferase expression normalized to *Renilla* luciferase. * indicates statistical significance by *t* test analysis ($p < 0.05$, $n = 3$). Inset: Sequence and location of modifications used in chemically modified siRNA.

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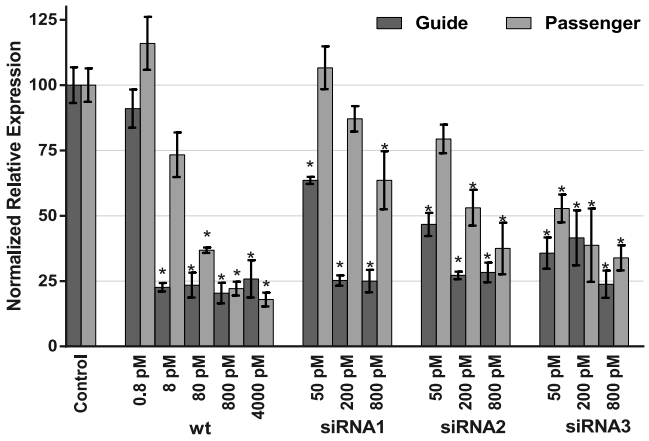

Figure 3. Reduction in the appropriate firefly luciferase variant mRNA due to guide and passenger strand activity. Guide and passenger strand target knockdown by wildtype and phenylethyl phosphate-modified siRNAs was measured by qPCR analysis. Target expression was normalized against the *Renilla* luciferase expression control. *indicates statistical significance by *t* test analysis ($p < 0.05$, $n = 3$).

modifications to encourage passenger strand uptake suggests that similar modifications may be well tolerated in the guide strand and warrants further investigation.

Many other phosphate modifications have been developed, yet few have been investigated in siRNA. Negatively charged backbone modifications such as the phosphorodithioate,[32] and neutral ones, such as triazole,[14] and amide linkages[13,26] have been used to improve serum stability and/or cell permeability. The location of these modifications also plays a major role in whether it is RNAi-activating or deactivating. For example, the placement of a single amide linkage at the 5′ terminal of the passenger strand was dramatically effective at improving strand selection.[33] siRNA **1** also carries a neutral linkage at the 5′ terminal of the passenger strand and may be selected in a similar manner. Therefore, electrostatic interactions between the first 5′ linkage and the RISC likely play a significant role in strand selection, which is not surprising given the phosphate dependent RNA−protein interactions identified at this location within the RISC.[34]

Finally, we examined the effect of nuclease stability on these siRNAs. It is well-known that RNAs exhibit high degradation rates in the presence of nuclease-rich environments.[35] Unmodified (**wt** siRNA) and modified siRNAs **1**−**5** were incubated in fetal bovine serum (FBS) for up to 4 h to analyze the degraded products in a time-dependent manner using polyacrylamide gel electrophoresis (Figure 4).

Unmodified **wt** siRNA degraded almost immediately, with almost no full-length siRNA remaining after 0.5 h. siRNA **1**, which contains the internal backbone modifications, also degraded almost immediately, with little remaining after 1 h. In contrast, siRNA **2**, which contains the phenylethyl backbone modification **dUxdU** (the deoxyuridine-based dinucleotide), directly at the 3′-end of the passenger strand, exhibited excellent nuclease stability up to 4 h. siRNA **3**, which contains the backbone modification **dUxdU** directly adjacent to a single deoxythymidine nucleoside residue on the 3′-end overhang, also exhibits enhanced nuclease stability compared to **wt** siRNA (intact siRNA viewed as long as 1 h). However, compared to siRNA **2**, this one is degraded more rapidly. Finally, siRNAs **3** and **4** contain the phenylethyl backbone modification **dTxdT** (the deoxythymidine-based dinucleotide) at the 3′-end and exhibit good nuclease stability up to 2 and 3 h, respectively. Thus, 3′-end phenylethyl phosphate backbone modifications offer enhanced nuclease resistance and thus may be used as a viable option for increasing the lifetime of siRNAs

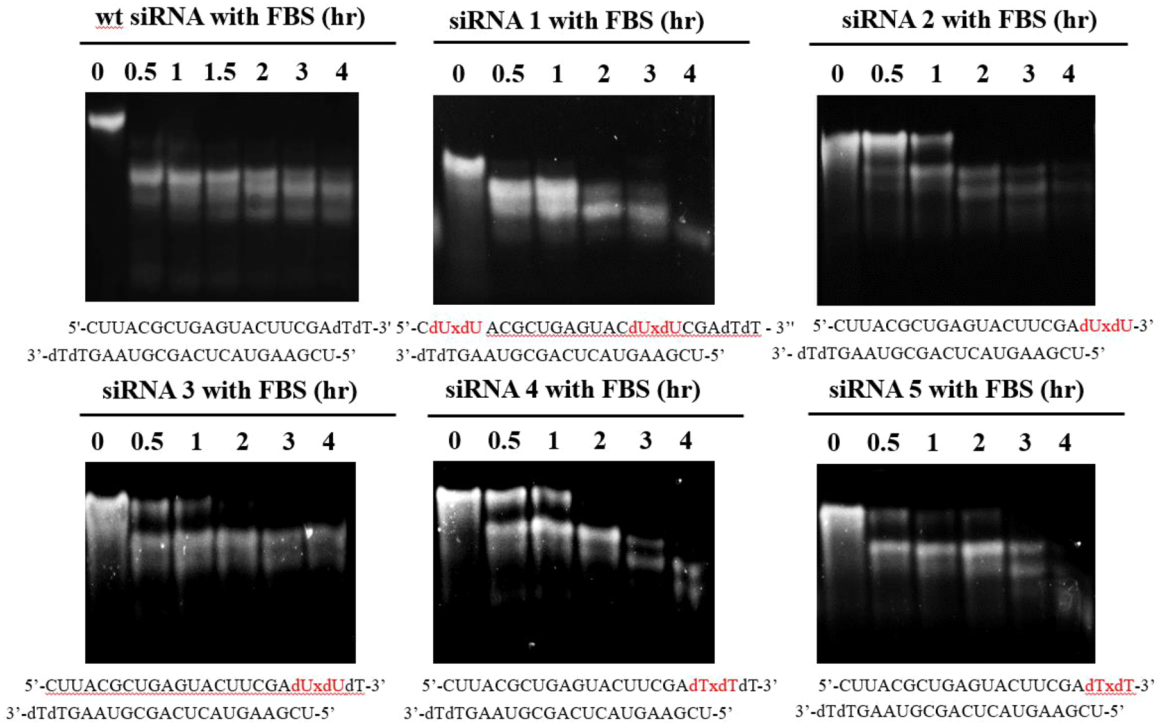

Figure 4. Nuclease stability assay. Samples were run on 20% nondenaturing polyacrylamide gel degradation products of siRNAs **wt** and **1**−**5** after incubation with 13.5% fetal bovine serum at 37 °C from 0 to 4 h.

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in the blood serum. Internal modifications (siRNA **1**) do not appear to offer a significant advantage in stability.

In conclusion, we are reporting the synthesis of a neutral phenylethyl phosphophosphate triester DMT-phosphoramidite using recently published methodology published from our lab that involves transesterification from tris (2,2,2-trifluoroethyl phosphate).[21] From this phosphoramidite, a small library of siRNAs bearing this modification at different positions of the passenger strand was generated. Excellent gene-silencing was observed as monitored using the Dual-Luciferase Reporter Assay. Furthermore, we examined the guide- and passenger-strand mediated gene silencing and observed that siRNA **1**, which contains two internal phenylethyl phosphate modifications improved the desired guide strand-mediated gene silencing, whereas the 3′-end modifications appeared to not offer any significant advantage in comparison to **wt** siRNA. In contrast, siRNAs **2**−**5**, which contained the 3′-end modifications, offered enhanced nuclease stability compared to internal backbone modifications (siRNA **1**). Phosphate triesters have been studied for many years, but to our knowledge these specific neutral phosphate triester compounds have not been tested for gene silencing or nuclease stability, and thus, we believe that this is the first type of investigation. The passenger strand was selected for modification because we wanted to separate the gene silencing effects from the nuclease stability effects. Our choice in using the *O*-phenylethyl phosphate was based on our desire to incorporate an aromatic hydrophobic functionality, while maintaining a relatively short aliphatic carbon chain to the phosphate backbone. Future work involves expanding and examining the synthesis of several different kinds of derivatives with variable length alkyl chains, and bulkier groups to further examine new structure−activity relationships.

Modification of the guide strand could have made silencing less effective, and therefore the siRNA overall would have had less utility than it currently does. This way, the silencing is effective, but the phosphate triester provides high nuclease resistance as well. This provides the benefit of selecting a phosphate triester for nuclease stability but then leaves the guide strand open to further potential modification if desired. Thus, it appears that the activity and stability of these novel backbone-modified siRNAs can be fine-tuned depending on its location within the passenger strand. Future directions include developing oligonucleotides with other triphosphate modifications such as small molecules that can further explore the structure−activity relationship of oligonucleotides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00232.

Synthetic procedures for compounds **5**−**14** (pages S3−S10), all procedures for oligonucleotide synthesis and characterization (pages S11−S12), procedures for nuclease stability testing (page S13), and procedures for testing gene silencing ability *in vitro* (pages S13−S14). In addition, $^{1}H/^{13}C/^{19}F/^{31}P$ NMR spectral data for compounds **5**−**14** (pages S18−S33) (PDF)

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Notes

The authors declare no competing financial interest.

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